PROCEEDINGS
OF THE
NATIONAL ACADEMY OF SCIENCES

Volume 32 July 15, 1946 Number 7

Copyright 1946 by the National Academy of Sciences

*ON NATURAL AUTO-ANTIBODIES AS EVIDENCED BY ANTIVENIN IN SERUM AND LIVER EXTRACT OF THE GILA MONSTER**

By Albert Tyler

William G. Kerckhoff Laboratories of the Biological Sciences, California Institute of Technology

Communicated June 3, 1946

In the course of experiments on the fertilizins and antifertilizins of sea-urchin eggs and sperm it was found[1] that an extract of eggs that had been deprived of their gelatinous surface coat was capable of agglutinating intact eggs and of forming a precipitate with the surface coat substance. Similar results have been obtained, although not consistently, with blood cells of mammals and with bacteria. On the basis of these and of various earlier reports in immunological literature that indicate a similar situation the view was proposed[1, 2] that all cells contain their own antibodies. The ability to demonstrate antigen-antibody like systems in a cell would, of course, depend upon use of appropriate extraction methods and upon the particular properties and amounts of the substances involved. Thus, for example, if surface substance were present in excess, an extraction procedure that involved destruction of the whole cell would permit interaction of the complementary surface and subsurface substances and the latter would not be found in the extract.

The finding of one antigen-antibody like system in a cell leads to the presumption that there may be others and to the view[1, 2] that such systems of complementary substances form the basis of cell structure. The synthesis of specific substances of large molecular size that comprise the structural elements of cells is thus conceived as occurring in a manner analogous to antibody formation. For the latter, there is now wide acceptance of the general views of Breinl and Haurowitz,[3] Alexander [4] and Mudd[5] involving an orienting influence of antigen, and Pauling[6] has formulated and experimentally supported[7] a specific mechanism based on the determination by antigen of the pattern of folding of polypeptide chains. This has been extended to the synthesis of specific substances and self-reproducing entities

(genes) in cells by Pauling and Delbrück[8] and Emerson.[9, 10] On a similar basis, schemes for the mode of action of genes and the possibility of inducing specific mutations by antibodies have been proposed and examined experimentally by Sturtevant[11] and Emerson.[9, 10]

The view[1] that all cells normally contain antibodies directed against certain of their constituents raises the possibility of deriving protective agents against pathogenic organisms directly by extraction of the organisms themselves rather than by the usual immunization procedures. The present author has made a number of attempts to obtain such protective auto-antibodies from pneumococci but these have, so far, been unsuccessful. Due to the factors mentioned above concerning possible extraction difficulties involved, the negative evidence cannot, as yet, be regarded as a sufficient basis for abandonment of the attempts. In the meantime, it appeared desirable to investigate other kinds of material from the same point of view. For this purpose a venomous reptile, the Gila monster, was selected. In general, venomous animals are resistant to homologous venom (see Calmette,[12] Noguchi,[13] Essex[14]) although the resistance is evidently[15] not absolute. Knowing this, many early workers have attempted to obtain auto-antivenins from the blood and organs, but with conflicting results[12, 13, 14]. More recently Rosenfeld and Glass[16] have shown that rattlesnake blood, although itself lethal for mice, neutralizes the hemorrhagic principle of the venom. Another report,[17] from the Serum Institute in Dorpat, Esthonia, is to the effect that viper serum loses its toxicity upon aging or heating, and is capable of neutralizing homologous venom. In the Gila monster, Cooke and Loeb[18] concluded that no antivenin was present in the serum. However, their data are not quite conclusive in that regard since, out of 17 mice that were given lethal doses of venom along with 1 ml. of serum, five survived. In the experiments reported here a definite protective action of serum and of liver extract is found.

Material and Methods.—Three Gila monsters of the species *Heloderma suspectum* were used. Two of these were injected with pilocarpine in order to induce copious secretion and exhaust the venom glands. The animals were each given an injection of 0.5 ml. of 1.3% pilocarpine on two consecutive days and the secretion collected each day shortly after the injection. The secretions were tested by intra-abdominal injection of mice. That collected on the first day proved lethal to mice in doses averaging 0.002 to 0.003 ml. while the second day's secretion was non-venomous in doses up to 0.2 ml. Soon after the second injection the animals were bled and venom glands, liver, pancreas, spleen and kidney removed. Later, serum was taken from the blood clots and the organs were washed and extracted with 10 volumes of saline by means of a Waring blendor and centrifugation at 2000 g. for 30 minutes. Venom, serum and organ extracts were also obtained from the uninjected third animal to serve as a check on possible in-

TABLE 1

TESTS OF VARIOUS SERA AND ORGAN EXTRACTS FOR ABILITY TO NEUTRALIZE HELODERMA VENOM

(Mice injected intra-abdominally with 1 ml. of mixtures containing 8 MLD of venom and dilutions of test material. Mixtures allowed to stand 15 minutes at room temperature. The asterisk designates tests of the toxicity of the material without venom.)

MATERIAL TESTED	AMOUNT, ML.	NO. OF MICE AT 4 DAYS	
		DEAD	ALIVE
*Serum of Heloderma #1 or #2	0.5	0	8
Serum of Heloderma #1 or #2	0.125 to 0.5	0	32
Serum of Heloderma #1 or #2	0.063	5	7
Serum of Heloderma #1 or #2	0.031	11	1
Serum of Heloderma #1 or #2	0.016	8	0
*Serum of Heloderma #3	0.5	0	4
Serum of Heloderma #3	0.25 and 0.5	0	24
Serum of Heloderma #3	0.125	6	6
Serum of Heloderma #3	0.063	8	4
Serum of Heloderma #3	0.031	10	2
Serum of Heloderma #3	0.016	3	1
*Serum of Rabbit	0.5	0	4
Serum of Rabbit	0.5	4	0
*Serum of Chuckwalla	0.5	0	4
Serum of Chuckwalla	0.063 to 0.5	20	0
Serum-Globulin of Heloderma #1	0.125 to 0.25	0	8
Serum-Globulin of Heloderma #1	0.063	3	1
Serum-Albumin of Heloderma #1	0.25	4	0
*Extract of Venom Gland of Heloderma #1	0.5	0	4
Extract of Venom Gland of Heloderma #1	0.5	7	1
Extract of Venom Gland of Heloderma #1	0.063 to 0.25	12	0
*Extract of Venom Gland of Heloderma #3	0.5	4	0
Extract of Venom Gland of Heloderma #3	0.125 to 0.5	12	0
*Extract of Liver of Heloderma #1	0.5	0	4
Extract of Liver of Heloderma #1	0.5	1	7
Extract of Liver of Heloderma #1	0.25	0	4
Extract of Liver of Heloderma #1	0.125	3	1
Extract of Liver of Heloderma #1	0.063	4	0
*Extract of Liver of Heloderma #3	0.5	0	4
Extract of Liver of Heloderma #3	0.5	7	1
Extract of Liver of Heloderma #3	0.25	5	3
Extract of Liver of Heloderma #3	0.125	5	3
Extract of Liver of Heloderma #3	0.063	7	1
*Extract of Pancreas of Heloderma #1	0.5	0	4
Extract of Pancreas of Heloderma #1	0.5	4	0
*Extract of Spleen of Heloderma #1	0.5	0	4
Extract of Spleen of Heloderma #1	0.5	4	0
*Extract of Kidney of Heloderma #1	0.5	0	4
Extract of Kidney of Heloderma #1	0.5	3	1

fluence of pilocarpine on the properties of these materials. In addition serum of the rabbit and of a non-venomous lizard, the chuckwalla (*Sauromalus ater*) were tested. All tests were made with white mice weighing about 28 g. They were kept for four days following injection. In no case did animals that survived the first day subsequently succumb and most deaths occurred within six hours. The venom was titrated in each set of experiments. The test dose employed in the experiments listed in table 1 contained 6 to 10 (av. 8) MLD. In these experiments venom and test material were mixed and kept at room temperature for 15 minutes before injection. Between sets of experiments the material was stored in the frozen state.

Results.—The site of synthesis of the venom in the Gila monster is not definitely known. On the supposition that it may be located in the venom gland, extracts of that organ were examined for the possible presence of antivenin. The pilocarpine-injected animal was first investigated since the heavy secretion of venom induced in it offered a more favorable opportunity for obtaining antivenin. However, the results (see table 1) were negative. The efficacy of pilocarpine in exhausting the gland of venom is illustrated by the fact that 0.5 ml. of the extract alone proved non-toxic. On the other hand, the venom gland extract of the non-pilocarpine-injected animal (Heloderma #3) proved quite lethal. The latter extract was also tested in mixtures with venom, for possible mutual neutralizing action, but here, too, the results were negative.

Since the location of the site of synthesis of the venom is not definitely known, it seemed desirable to examine serum and extracts of other organs for possible venom-neutralizing action. As the results presented in table 1 show, the Heloderma's serum and liver extract proved to be effective. In most of these tests both the venom and serum or extract employed were derived from the same individual. The protective action is exhibited by serum of both the pilocarpine-injected (#1 and #2) and the non-injected (#3) animals, and by liver-extract of a pilocarpine-injected animal (#1). Pilocarpine alone (unlisted tests) gives no protection. Complete protection against 8 MLD of venom is obtained with 0.125 to 0.25 ml. of serum and 50% protection with 0.063 to 0.125 ml. While the serum of the pilocarpine-injected animal has given somewhat better protection than that of the non-injected animal, more data would be required before any significance can be attached to the difference. In the case of the liver extracts the difference appears to be greater, but in view of the difficulty of maintaining identical treatment in preparation of the extract, it cannot as yet be concluded that liver from a non-pilocarpine-injected Heloderma is incapable of yielding much protective material. It may also be noted that the latter extract gives somewhat irregular results upon dilution. This may be due to a slow release of protective material from particulate matter in the extracts upon dilution or to some other unknown factor.

The tests with serum of the rabbit and of the chuckwalla revealed no neutralizing action on the part of these materials. Likewise the extracts of pancreas, spleen and kidney of Heloderma were found to be incapable of neutralizing any significant amount of venom.

Globulin and albumin fractions were prepared from Heloderma serum by precipitation with ammonium sulphate. The globulin fraction, adjusted to original serum concentration, was found to possess approximately the same neutralizing power as whole serum while the albumin exhibited no protective action.

Some tests (not listed in the table) were made in which the mice (16) received injections of Heloderma serum at various times after the injection of venom. With 0.125 ml. of serum complete protection was obtained at 3 to 6 minutes, partial protection at 10 to 12 minutes and no protection at 13 to 15 minutes.

Discussion.—The present results do not constitute direct evidence for the auto-antibody concept, as stated above, since for that purpose, it would be necessary to demonstrate the presence of auto-antivenin in the cells in which venom is synthesized. However, they do furnish some support for it, particularly since the expectation of finding an auto-antivenin somewhere in the animal was based on that view. The results may, then, be interpreted in the following manner. Venom and antivenin, in combination, are both assumed to be liberated into the blood stream from the organ, perhaps the liver, in which they are synthesized. The venom gland then effects a separation of the two so that venom accumulates in the gland from which it is later secreted, while the antivenin is left in the blood. Various methods of testing this and other similar interpretations suggest themselves and these will constitute the subject of further investigations that are planned along this line.

Several earlier investigations were mentioned in the previous articles[1, 2] as indicative of natural auto-antibodies. Since then others have appeared that point in the same general direction. Perhaps the most pertinent is the finding by Kidd and Friedewald[19] that normal rabbit serum reacts with a sedimentable constituent obtained in extracts of various organs of the same animal. This can be interpreted as due to the release into the blood of substances that are formed in connection with the sedimentable constituents of the tissues involved and that have configurations complementary to these constituents. On the basis of the auto-antibody concept one would, in fact, expect to be able to find complementary substances for all of the large molecular constituents of serum, located in the tissues where the latter are formed. Recent evidence,[20] pointing to the lymphocytes as the source of gamma-globulin, leads to the expectation of finding natural anti-globulin therein.

Auto-antibodies that are presumed to arise from an immunization process

are found in the serum of individuals having various diseases, such as: syphilis, infectious mononucleosis, yellow fever, acute hepatitis, malaria and atypical pneumonia.[21] In these cases it is generally believed that the pathogenic organism furnishes the foreign protein that serves to form a complete antigen with some constituent of the host tissue or that the organism possesses an antigen that is serologically similar to the host tissue constituent. On the basis of the natural auto-antibody concept a different interpretation may be offered. The infectious agent is assumed to release from the tissue undergoing destruction, pairs of mutually complementary substances, of which one is quickly removed from the circulation while the other remains there for a time. Thus, in syphilis, it would be presumed that the spirochaete produces a lipo-protein-splitting enzyme, such as has been reported[22] in other organisms, and that the lipid is removed from the blood leaving behind the complementary protein which is identified as the Wassermann reagin.

Naturally occurring substances that react with hormones or enzymes of the same animal have been described.[23] It seems possible that these, too, may represent auto-antibodies. There is also some evidence[24, 25] that antibacterial proteins such as lysozyme and diplococcin are derivable from the species of organism on which they act. It has, in fact, been suggested [24] that "Such enzymes, which in high concentration partly or completely lyse the organisms from which they are derived, are probably concerned with bacterial multiplication." Autolytic enzymes, such as that of the pneumococcus,[26] may belong to the same category.

While these various suggestions are largely of speculative nature, they have the advantage of being subject to experimental attack and offer the possibility of contributing to the analysis of cell growth and differentiation as well as to the practical problems of protection against infection and abnormal growth.

Summary.—Serum of Heloderma was found to be capable of neutralizing the venom of the same animal. Auto-antivenin was also found in extract of liver of a pilocarpine-injected Heloderma but not in extracts of venom gland, pancreas, spleen and kidney of this animal, nor was antivenin found in the serum of the rabbit and the chuckwalla. The antivenin was obtained in the globulin fraction of the Heloderma serum. The bearing of these results on the natural auto-antibody concept, previously proposed, is discussed and applications of this concept to various immunological and general biological problems are indicated.

* With the aid of a grant from The Rockefeller Foundation. The author is indebted to Miss Margaret L. Campbell for technical assistance and to Professors A. H. Sturtevant and S. Emerson for their interest and advice.

[1] Tyler, A., *Proc. Nat. Acad. Sci.*, **26**, 249–256 (1940).

[2] Tyler, A., *West. Jour. Surg., Obstet., & Gynec.*, **50**, 126–138 (1942).

[3] Breinl, F., and Haurowitz, F., *Z. physiol. Chem.*, **192**, 45–57 (1930).
[4] Alexander, J., *Protoplasma*, **14**, 296–306 (1932).
[5] Mudd, S., *Jour. Immunol.*, **23**, 423–427 (1932).
[6] Pauling, L., *Jour. Amer. Chem. Soc.*, **62**, 2643–2657 (1940).
[7] Pauling, L., and Campbell, D. H., *Jour. Exptl. Med.*, **76**, 211–220 (1942).
[8] Pauling, L., and Delbrück, M., *Science*, **92**, 77–79 (1940).
[9] Emerson, S., *Proc. Nat. Acad. Sci.*, **30**, 179–183 (1944).
[10] Emerson, S., *Ann. Missouri Bot. Garden.*, **32**, 243–249 (1945).
[11] Sturtevant, A. H., *Proc. Nat. Acad. Sci.*, **30**, 176–178 (1944).
[12] Calmette, A., *Venoms*, Chap. 10, John Bale & Sons, London, 1908.
[13] Noguchi, H., *Snake Venoms*, Publ. No. 111, Carnegie Institution of Washington, 1909.
[14] Essex, H. E., *Physiol. Rev.*, **25**, 148–170 (1945).
[15] Nichol, A. A., Volney, D., and Peck, L., *Copeia*, No. 4, Dec. 27, 211–213 (1933).
[16] Rosenfeld, S., and Glass, S., *Amer. Jour. Med. Sci.*, **199**, 482–486 (1940).
[17] Peterson, H., and Kõivastik, Th., *Zeitschr. Immunitätsforsch.*, **102**, 324–331 (1942).
[18] Cooke, E., and Loeb, L., Chap. II in *The Venom of Heloderma* by L. Loeb, Publ. No. 177, Carnegie Institution of Washington, 1913.
[19] Kidd, J. G., and Friedewald, W. F., *Jour. Exptl. Med.*, **76**, 543–578 (1942).
[20] Kass, E. H., *Science*, **101**, 337–338 (1945); Harris, T. N., Grimm, E., Mertens, E., and Ehrich, W. E., *Jour. Exptl. Med.*, **81**, 73–84 (1945); Ehrich, W. E., Harris, T. N., and Mertens, E., *Ibid.*, **83**, 373–382 (1946).
[21] See: Landsteiner, K., *The Specificity of Serological Reactions*, Harvard Univ. Press, 1945; Weil, A. J., *Bact. Rev.*, **5**, 293–330 (1941); Neurath, H., *et al.*, *Science*, **101**, 68–69 (1945); Eaton, M. D., Murphy, W. D., and Hanford, V. L., *Jour. Exptl. Med.*, **79**, 539–557 (1944); Peterson, O. L., Ham, H. T., and Finland, M., *Science*, **97**, 167 (1943); Thomas, L., Curnen, E. C., Mirick, G. S., Ziegler, J. E., Jr., and Horsfall, J. E., Jr., *Proc. Soc. Exp. Biol. & Med.*, **52**, 121–125 (1943); Turner, J. C., and Jackson, E. B., *Brit. Jour. Exptl. Path.*, **24**, 121–126 (1943); Heidelberger, M., and Mayer, M. M., *Science*, **100**, 359–360 (1944).
[22] Crook, E. M., *Brit. Jour. Exptl. Path.*, **23**, 37–55 (1942).
[23] See: Richardson, R., *Proc. Soc. Exp. Biol. & Med.*, **38**, 874–875 (1938); Northrop, J. H., *Crystalline Enzymes*, Columbia Univ. Press, 1939; Sevag, M. G., *Immuno-Catalysis*, Charles C Thomas, Baltimore, 1945; Horwitt, M. K., *Science*, **101**, 376–377 (1945).
[24] Meyer, K., *Science*, **99**, 391–393 (1944); Meyer, K., Palmer, J. W., Thompson, R., and Khorazo, D., *Jour. Biol. Chem.*, **113**, 479–486 (1936).
[25] Oxford, A. E., *Biochem. Jour.*, **38**, 178–182 (1944).
[26] Avery, O. T., and Cullen, G. E., *Jour. Exptl. Med.*, **38**, 199–206 (1923); see also Dubos, R. J., *The Bacterial Cell*, Harvard Univ. Press, 1945.

A NEW TYPE OF ISOLATING MECHANISM IN DROSOPHILA

By J. T. Patterson

Genetics Laboratory, University of Texas

Communicated June 10, 1946

While working on the general problem of sexual isolation in the genus Drosophila, the writer discovered a mechanism which forms a very effective barrier against the exchange of genes between certain members of this genus. It was first detected in the cross *D. buzzatii* ♀ × *D. arizonensis* ♂, two members of the mulleri subgroup of species. It was later found to be operative between other members of this subgroup, as well as between species belonging to other groups. The phenomenon stems from a reaction of the vagina that almost immediately follows copulation, and may be designated the *insemination reaction*. Within a very short time after mating, the vagina begins to enlarge and soon takes on an edematous-like condition, increaseing to three or four times the normal size of this organ in virgin females.

This change occurs in both intraspecific and interspecific crosses, but in intraspecific crosses (homogamic matings) the vagina returns to its normal size in the course of a few hours, while in interspecific crosses (heterogamic matings) it may remain swollen for several days and undergo deleterious changes which prevent it from carrying on its normal functions. In extreme cases, the reaction is so severe that eggs descending from the median oviduct may disintegrate in the vaginal cavity, or, if laid, they are not inseminated and are sometimes abnormal.

The *buzzatii-arizonensis* combination may be used to illustrate this reaction and its consequences. In previous publications from this laboratory, the cross *D. buzzatii* ♀ × *D. arizonensis* ♂ was designated as "incompatible." This term was employed in its usual sense to indicate that such test crosses do not result in the production of offspring. Recent studies on sexual isolation now reveal that the failure to produce hybrids in this cross is not due to the lack of insemination, but is the result of the insemination reaction that follows a successful copulation. When *buzzatii* females were crossed to *arizonenis* males in small mass cultures of ten females and ten males, 88% of the females were found to have been inseminated after an exposure of ninety-six hours, as shown by the presence of sperm in their ventral receptacles. We have tested this cross repeatedly but have never been able to obtain hybrids of any kind, either adults, pupae or larvae.

In studying this phenomenon, we have followed the plan of placing one virgin female with five males in a culture vial, and as soon as copulation had occurred the males and female were separated. Such females were dissected at given intervals of time and their reproductive tracts examined

for the presence of sperm and any changes that may have occurred in the vagina.

In homogamic matings the vagina begins to enlarge in about four minutes after copulation, and continues to enlarge until it reaches the maximum size at the end of about one hour. Within twenty-five to thirty minutes after copulation the vagina turns slightly opaque and continues to become more and more so up to the end of the second hour. It then begins to show signs of clearing, and within five or six hours its opacity has completely disappeared. It returns to its normal size and semi-transparent condition by the end of eight hours.

The behavior of the sperm following insemination is a matter of interest. Within five minutes after copulation the sperm begin moving along the ventral side of the vaginal cavity to the point of origin of the ventral receptacle and soon start entering that organ. They continue to pass into the receptacle until it may become packed with these elements. Since in species belonging to this subgroup, the sperm rarely ever enter the spermathecae, there is frequently an excess of sperm left in the swollen vagina. Eventually, these are expelled, together with the rest of the contents of the vagina, in the form of whitish droplets which are easily seen on the surface of the food and which may be removed to a slide and examined under the microscope.[1] Undoubtedly the voiding of the contents of the vagina accounts for the return of that organ to a normal condition by the end of the eighth hour. A majority of these females will, in due time, lay eggs and produce offspring without further inseminations.

In heterogamic matings the behavior of the sperm and the reaction of the vagina are at first similar to those occurring in intraspecific crosses, but certain significant differences soon appear. In the first place, the insemination reaction occurs somewhat more rapidly and the opacity of the vagina is more pronounced. Moreover, the sperm are slower in reaching the mouth of the ventral receptacle and do not begin entering that organ until about forty-five minutes after copulation. A large mass of sperm frequently collects about the opening to the receptacle, and the sperm have difficulty in their attempts to enter its lumen. A few sperm are usually present in the proximal end of the receptacle by the end of two hours, and by the end of three hours a few are at the distal end. While the quantity of sperm which finally reaches this position varies considerably, yet this amount is always very much less than is the case in homogamic matings. In one case, only two spermatozoa were present at the distal end, and in another only six. In practically all cases the sperm are restricted to the distal one-third or one-fourth of the ventral receptacle. In contrast to this condition, the receptacle always contained many sperm in homogamic matings and was frequently recorded as being "full" or "solid" with them.

In homogamic matings the vagina may soon return to a normal condi-

tion, as stated above, but in heterogamic matings its swollen condition may last for several days, accompanied by certain significant changes. One of the first differences noted is a change in color. At the end of the third hour after copulation the vagina turns slightly brownish and remains so up to about the twenty-fifth hour, when it often appears blackish under transmitted light. By forty hours the contents of the vagina become localized and sharply delimited, occupying the antero-dorsal part of the cavity. As time goes on this *reaction mass* becomes reduced in size and pear-shaped with the smaller end directed posteriorly. Further reduction may occur until its size does not constitute more than one-fourth the volume of the vagina. In some cases the reaction mass may undergo disintegration and entirely disappear, although usually traces of it can be detected. Even in such cases the vagina is left abnormal in appearance. In the *buzzatii-arizonensis* cross the reaction mass is usually present in females dissected on the seventh day after copulation.

Since only a part, and usually a very small part, of the sperm received by the female ever succeeds in entering the ventral receptacle, the question arises as to what becomes of those remaining in the vagina. Motile sperm are easily seen in this organ and have been detected in practically every case up to about the seventh hour after copulation. They have not been observed after this period, although the vagina of one female of the eighth hour contained a group of non-motile sperm located on the ventral side of its cavity. No evidence was obtained that the excess sperm had been expelled as in homogamic matings, but this remains as a possibility. It may be that these sperm are absorbed by the reproductive tract, as has been suggested for certain other insects.[2]

The sperm in the distal end of the ventral receptacle remain alive for at least 160 hours, and perhaps longer. There is, however, no evidence in the *buzzatii-arizonensis* cross that these sperm ever inseminate the eggs. The first egg discharged from the ovary in this series of dissections was forty-eight hours after copulation. It was just entering the median oviduct when first seen. One female dissected at fifty-six hours had laid five eggs, and two dissected at seventy-two hours had each laid one egg. These eggs were checked by the smear technique,[3] but none was found to contain sperm. The females had laid a considerable number of eggs by ninety-six hours, but most of these were transparent, like eggs laid by virgin females. A total of 105 of these eggs were checked for the presence of sperm and all were found not to have been inseminated. Some of the eggs break down and go to pieces in the vagina, and its cavity may become full of their debris. In one case, parts of three eggs were found in the vaginal cavity, and eggs in various degrees of disintegration were sometimes observed.

The intraspecific matings of nine different species of the mulleri subgroup have been examined for the presence of the insemination reaction and with-

out exception all showed that it occurred in them. In interspecific crosses between these species, the reaction was found to occur in all combinations in which copulation had been successful, irrespective of whether or not such matings had resulted in the production of hybrids. Successful copulations did not take place in a majority of the combinations tested, but in all crosses in which it did occur, the history of the insemination reaction was essentially the same as outlined above for the *buzzatii-arizonensis* cross. In the few cases in which hybrids were produced, the reproductive tract of the female had recovered sufficiently to allow some of the eggs to become inseminated. In most crosses, however, the number of hybrids produced was relatively small, and these were sometimes abnormal or else the zygotes never developed beyond the mid-larval stage. The most successful cross, in so far as the production of hybrids is concerned, was between the two most closely related forms, *D. mojavensis* and *D. arizonensis*.

The hybrids from the cross *D. mojavensis* ♀ × *D. arizonensis* ♂ are fertile, and pair mating tests show that approximately 75% of the females produce offspring, although the average number of individuals per culture is relatively low. In one experiment, six-day-old *mojavensis* females were crossed to *arizonensis* males of the same age in small mass matings, and four days later the females were dissected and their seminal receptacles examined. It was found that 93% of these females had been inseminated, and of this number, the vagina was clear and normal in twenty-seven cases, clear and nearly normal in thirty-three others, and opaque and abnormal in the thirty-three remaining specimens. The ventral receptacles of all the females of groups one and two contained motile sperm, and these are the females that are responsible for the production of the hybrids. It is doubtful if the vagina in any of the females belonging to the third group would ever recover sufficiently to enable them to lay fertile eggs.

This brief account of the insemination reaction in the mulleri subgroup raises the question as to its possible occurrence in other species of the genus. In past studies on members of the virilis group we had, from time to time, observed this reaction in interspecific crosses, but had not realized its significance. With the knowledge gained in studying the mulleri series, we reexamined the virilis group and found that the reaction also occurs among members of that group. However, the insemination reaction is rather inconspicuous in homogamic matings and could be overlooked. The reaction is quite obvious in the heterogamic matings, although not so striking as in the mulleri series.

Mr. Marshall R. Wheeler has undertaken a study of all available species of Drosophila in the laboratory, with the view of determining the extent of the insemination reaction among other members of the genus. He studied the intraspecific crosses only and used the method given above for obtaining a timed series of stages. In the list given below, members of the virilis and

mulleri series were examined by the writer, all others were studied by Mr. Wheeler, to whom I am indebted for the privilege of including his results in advance of publication. The several species examined are given in their systematic order as outlined by Sturtevant.[4] The terms present and absent are used to indicate whether or not the insemination reaction was found to occur in the different species. The list is not complete, but gives all forms thus far examined.

Subgenus *Pholadoris:* present in *D. victoria.* Subgenus *Sophophora:* 1. saltans group, absent in *D. prosaltans.* 2. willistoni group, absent in *D. nebulosa.* 3. melanogaster group, absent in *D. melanogaster.* 4. obscura group, absent in *D. pseudoobscura.* Subgenus *Drosophila:* 1. quinaria group, present in *D. transversa, D. innubila, D. subpalustris, D. suboccidentalis* and *D. subquinaria.* 2. guttifera group, present in *D. guttifera.* 4. virilis group, present in *D. virilis, D. americana* and *D. texana.* 6. tripunctata group, absent in *D. tripunctata.* 7. funebris group, present in *D. funebris.* 8. repleta group, absent in *D. hydei;* present in *D. mercatorum, D. pararepleta, D. hexastigma* and *D. gibberosa.* 8a. mulleri subgroup, *D. anceps, D. aldrichi, D. arizonensis, D. buzzatii, D. hamatofila, D. mojavensis, D. peninsularis, D. mulleri* and *D. ritae,* present in all nine. 10. melanica group, present in *D. melanica.* 13. cardini group, absent in *D. cardini.* 14. immigrans group, present in *D. immigrans.* Miscellaneous forms, present in the two subspecies, *D. pallidipennis pallidipennis* and *D. p. centralis.*

This list shows that the insemination reaction was found to occur in twenty-eight of the thirty-five species examined. On the basis of this study, it seems safe to predict that it is present in all members of the quinaria and virilis groups and the mulleri subgroup, but probably absent in all members of the subgenus *Sophophora.* The repleta group presents an interesting situation. The reaction was found to be present in thirteen of the fourteen species examined, with *D. hydei* representing the exception. There is incomplete evidence that some of the forms closely related to *hydei* also do not have the reaction. If this turns out to be true, it will be possible to divide the large repleta group into two main divisions, one with and one without the reaction.

It is legitimate to speculate on the nature of the insemination reaction and its possible rôle in Drosophila speciation. It is well at first to restate briefly just what happens after copulation has taken place. The introduction of the semen with its contained spermatozoa into the vagina is followed almost immediately by a reaction of the mucous membrane which secretes a relatively large amount of fluid into the cavity. This is what brings about the characteristic swelling of the vagina. This membrane must be hypersensitive to the foreign protein, and, since the reaction occurs after the first copulation, its hypersensitiveness must be an inherited character. Whether the spermatozoa *per se,* or the semen, or both are responsible for calling forth the

reaction is a matter of considerable interest. We have observed, from time to time, that in some crosses the male had failed to deliver spermatozoa with the semen at the time of copulation, and yet the typical insemination reaction had occurred. The most careful examination of the freshly formed reaction mass in such cases failed to disclose the presence of sperm. Moreover, no evidence was found of any form of lysis that might have been responsible for the absence of sperm.[5]

A more convincing line of evidence that the sperm are not responsible for the reaction is seen in the results obtained in backcross matings of sterile F_1 males. These males have slightly smaller testes than normal, and spermatozoa are never formed in them. The F_1 males from the cross *arizonensis* ♀ × *mojavensis* ♂ are of this type, and they will sometimes mate with either *arizonensis* or *mojavensis* females. Their sperm-free semen always brings about the typical insemination reaction. It is, therefore, certain that the presence of sperm in the semen is not necessary for the induction of the reaction.

Another point of interest is whether the insemination reaction will be repeated in the vagina of a female which has mated a second time. The answer to this question will depend on the species. In the first place, in forms which do not show the reaction, copulation may occur two or more times, and in some species at relatively short intervals. In species which show a weak reaction (e.g., virilis group), the female may remate two or more times at intervals of several hours, and a reaction of about the same intensity as the first occurs after each mating. Finally, in species in which the reaction is severe the female may never remate, but if she does, it is only after a long period of time, but here too the reaction occurs, provided the copulation has been successful.

Three possible functions may be suggested for the insemination reaction. The first of these refers to its occurrence in homogamic matings. It may have the effect here of preparing the reproductive tract for the fertilization mechanism which is to follow. It should be pointed out, in this connection, that even in forms which show no visible reaction there still may be a change in the mucous membrane which has the same effect. Unfortunately, this suggestion cannot be tested experimentally.

A second possible rôle of the insemination reaction has a more direct bearing on the problem of speciation. In species which do not have this reaction, the female may copulate two or more times in intraspecific matings. In contrast to this, the female usually mates but once in species showing a strong reaction. In the first instance the male in the population could, and probably would, mate with the same female more than once, instead of fertilizing additional females. In the second instance the male would be forced to copulate with more than one female, if he were to mate more than a single time. This might be an advantage in a population with a restricted

number of males. If the insemination reaction arose as a mutation, irrespective of any selective value it might have to the species as a whole, it would spread throughout the population. This is because virgin females might be inseminated by males either with or without the factor. Females fertilized by males not carrying the factor may be remated by both kinds of males. However, a female inseminated by a male with the factor is much less likely to be remated. Hence, a female mated by a male not carrying the factor may have progeny by several males, but a female carrying the factor will ordinarily have progeny by only one male. This is a very interesting example of how a mutation, even though it be non-adaptive, would replace its alleles in the population.

A third rôle is revealed in interspecific crosses among forms which have the insemination reaction, for here it has the effect of either reducing the exchange of genes, or preventing such exchanges altogether. The *buzzatii-arizonensis* cross is an excellent example of the complete elimination of gene transfer. Sexual isolation in this cross is weak and copulation occurs almost as frequently (88%) as in the intraspecific cross (97%), and yet, as a result of the insemination reaction, the females are unable to produce offspring.

In conclusion, it should be pointed out that the widespread occurrence of this reaction in the genus would suggest that it may be present in forms other than Drosophila. It is possible that the results reported above may have some bearing on the controversial point regarding the effect of sperm injections on the development of the reproductive tract in immature mammals.[6, 7]

It is a pleasure to acknowledge the excellent assistance given by Miss Johanna Blumel who cultured and collected the flies used in the experiments, and by Mrs. Sarah B. Martin who made most of the dissections.

[1] In 1942 Dr. Wilson S. Stone and the writer observed this same habit in females of the virilis group.

[2] Cragg, F. W., *Ind. J. Med. Res.*, **8**, 32–39 (1920); **11**, 449–473 (1923).

[3] Patterson, J. T., *Science*, **101**, 156 (1945).

[4] Sturtevant, A. H., *Univ. of Tex. Publ.*, **4213**, 7–51 (1942).

[5] I am indebted to Dr. V. T. Schuhardt, of the department of Botany and Bacteriology, for valuable suggestions in preparing this account on the nature of the insemination reaction.

[6] Green-Armytage, V. B., *Proc. Roy. Soc. Med.*, **36**, 105 (1943).

[7] Bacsick, Sharman, and Wyborn, *J. Obstet. Gynaec. Brit. Emp.*, **52**, 334 (1945).

HYBRIDIZATION BETWEEN RANA PALUSTRIS AND DIFFERENT GEOGRAPHICAL FORMS OF RANA PIPIENS

BY JOHN A. MOORE

BARNARD COLLEGE, COLUMBIA UNIVERSITY AND THE AMERICAN MUSEUM OF NATURAL HISTORY

Communicated June 11, 1946

In intraspecific crosses among different populations of *Rana pipiens* Schreber, hybridization leads to progressively greater embryonic defects with increasing latitudinal distance between the parent populations. Experiments have been performed with individuals from many localities in the eastern and central portions of the United States with the following results.[1] Normal or very slightly abnormal embryos are formed by crossing adults of these localities: Wisconsin × Vermont; Vermont × New Jersey; Vermont × Oklahoma; New Jersey × Louisiana; Louisiana × Ocala, Florida; Ocala, Florida × Englewood, Florida; Ocala, Florida × Texas. In crosses between extreme northern and southern individuals, such as Vermont × Florida or Wisconsin × Texas, the hybrids are very abnormal. If the female parent is from the north and the male from the south, the hybrids exhibit marked retardation in rate of development, extreme head enlargement and pronounced circulatory system defects. In the reciprocal cross, southern female and northern male, retardation in rate, reduction in head size with frequent fusion of olfactory pits and eyes, and the absence of a mouth are the typical abnormalities. The mortality varies in different experiments but is usually high.

It has been established that crosses between *Rana pipiens* from Vermont and *Rana palustris* Le Conte result in healthy hybrids which can be carried through metamorphosis.[2] Therefore, a Vermont *pipiens* can be crossed sucessfully with a different species but not with southern members of its own species. This unusual situation indicated that the results of crosses between *Rana palustris* and different geographical forms of *Rana pipiens* would be of interest. To secure data on this subject the following crosses were made:

*Vermont *pipiens* ♀ × *palustris* ♂ (5)
**palustris* ♀ × Vermont *pipiens* ♂ (5)
*Wisconsin *pipiens* ♀ × *palustris* ♂ (2)
**palustris* ♀ × Wisconsin *pipiens* ♂ (1)
**palustris* ♀ × Oklahoma *pipiens* ♂ (1)
Englewood, Florida *pipiens* ♀ × *palustris* ♂ (1)
palustris ♀ × Englewood, Florida *pipiens* ♂ (1)
*New Jersey *pipiens* ♀ × *palustris* ♂ (1)
**palustris* ♀ × New Jersey *pipiens* ♂ (1)
*Ocala, Florida *pipens* ♀ × *palustris* ♂ (1)
**palustris* ♀ × Ocala, Florida *pipiens* ♂ (1)
*Texas *pipiens* ♀ × *palustris* ♂ (1)
**palustris* ♀ × Texas *pipiens* ♂ (1)

All of the *Rana palustris* used in these experiments were collected in Massachusetts. In each type of cross marked with an asterisk, transformed young were secured. The figure in parentheses following each cross gives the number of crosses made. In all experiments in which the hybrids were raised to metamorphosis, it was found that the pigment pattern of the young was intermediate to that of the parents. There was no evidence of differential mortality between hybrids and controls in any experiment. In the Englewood, Florida *pipiens* ♀ × *palustris* ♂ cross, some embryos were kept until they were 40 mm. in length. They were then discarded, though normal. In the reciprocal cross, the embryos were also normal but no attempt was made to raise them to metamorphosis.

Crosses between Vermont *pipiens* and *palustris* have been described previously.[2] The rate of development of the hybrids was intermediate between that of the parents. No evidence of morphological defect was observed at any time during development.

In crosses between Wisconsin *pipiens* and *palustris*, the hybrids exhibited no structural defects and some were raised to metamorphosis. The rate of development was not determined. The burnsi mutant[3, 4] of *pipiens* was used in these experiments.

The *palustris* ♀ × Oklahoma *pipiens* ♂ hybrids were structurally normal. The rate of development was not determined. Some of the hybrids were carried through metamorphosis.

The crosses between *palustris* and Englewood, Florida *pipiens* are of interest as the Florida individuals give very abnormal hybrids with northern populations of *pipiens*. The Englewood, Florida *pipiens* ♀ × *palustris* ♂ hybrids were structurally normal. The rate of development was not determined. As noted before, these embryos were kept until they were tadpoles 40 mm. in length. The *palustris* ♀ × Englewood, Florida *pipiens* ♂ hybrids were structurally normal and developed at the same tempo as the maternal controls. In a parallel experiment some sperm of this same Florida *pipiens* male was used to fertilize Vermont *pipiens* eggs. The resulting intraspecific hybrids had greatly enlarged anterior ends, defective circulatory systems, and were markedly retarded in rate of development. The contrast between normal interspecific and abnormal intraspecific hybrids was striking.

The same *palustris* female was used in experiments with New Jersey, Ocala, Florida and Texas *pipiens* males. The hybrids with the New Jersey and Florida males developed normally. There was a possible acceleration in rate of development in the *palustris* ♀ × New Jersey *pipiens* ♂ hybrids during neural fold stages but later these embroyos were identical with the maternal controls. The *palustris* ♀ × Texas *pipiens* ♂ hybrids were retarded approximately ten per cent when the controls were in stage 20.[5] In addition the anterior end of these embryos showed a slight, but definite,

enlargement (this type of defect is observed in close latitudinal crosses of *Rana pipiens*). Embryos from these three crosses were raised to metamorphosis.

The cross, New Jersey *pipiens* ♀ × *palustris* ♂, resulted in morphologically normal embryos. The rate of development was not measured. The embryos were raised to metamorphosis.

The Ocala, Florida *pipiens* ♀ × *palustris* ♂ hybrids developed at a rate intermediate to that of the two parental species. These embryos were structurally normal and some were raised to metamorphosis. In a parallel experiment, eggs of this same female were fertilized with Vermont *pipiens* sperm. These intraspecific hybrids were retarded in development and had reduced anterior ends with fused olfactory pits.

The Texas *pipiens* ♀ × *palustris* ♂ hybrids were retarded (as were the reciprocal hybrids) and the anterior end appeared to be slightly reduced in size. Some of these embryos were raised to metamorphosis. Eggs from this same female were fertilized with New Jersey and Vermont *pipiens* sperm. Both groups of intraspecific hybrids were retarded in rate and were morphologically abnormal. The defects were most apparent in the Texas *pipiens* ♀ × Vermont *pipiens* ♂ embryos, many of which lacked a mouth. Once again gametes from the same individual were found to be more compatible with gametes of a different species than with those of the same species.

In summary, all of the crosses between *Rana palustris* and different populations of *Rana pipens* (except Texas) give normal hybrids. The defects noticed in crosses with Texas *pipiens* are so slight that they would not have been detected without close study.

The inviability observed in some *Rana pipiens* intraspecific crosses is thought to be due to complementary lethal genomes.[1] If such is the case, the type of incompatibility encountered when two complementary lethal genomes are present in the same individual would probably not result if either of these genomes was combined with a third genome. In the latter case, the genomes might be compatible, or if incompatibility was present it would probably be due to interaction of factors entirely different from those leading to inviability in the original case. The data cited in this paper indicate that the *Rana palustris* genome does not combine, with a resulting lethal effect, with any of the *Rana pipiens* genomes tested.

A somewhat similar case, of inability of individuals to cross with members of the same species coupled with the ability to cross with members of a different species, has been reported in cotton.[6, 7] The "crumpled" condition of the hybrids formed in certain crosses is due to the combined action of complementary lethal genes Cp_a and Cp_b. Cp_a is found in only one strain of *Gossypium arboreum*. In all other strains of this species, and in *Gossypium herbaceum*, this locus is represented by cp_a. Cp_b and cp_b occur with about

equal frequency in various strains of *Gossypium arboreum* and *Gossypium herbaceum*. An intraspecific cross, between an individual of *Gossypium arboreum* homozygous for cp_a and Cp_b with an individual of another strain homozygous for Cp_a *and* cp_b, would result in the "crumpled" condition. An interspecific cross involving either of these individuals and *Gossypium herbaceum* homozygous for cp_a and cp_b would result in normal progeny.

Rana palustris occurs in the eastern parts of the United States and Canada. Its range of distribution is entirely encompassed by the range of *Rana pipiens* The Vermont, Wisconsin, New Jersey and Oklahoma localities mentioned in these experiments are within the ranges of both species. In the Louisiana, Texas, Ocala Florida and Englewood, Florida localities, *Rana pipiens* is found—but not *Rana palustris*. In the localities where the two species occur together, natural selection has not resulted in the development of hybrid inviability as an isolating mechanism. It is of interest to note that the one strain of *Rana pipiens* which shows even slight incompatibility with *Rana palustris* is found in a locality in which only the former is present. The primary isolating mechanisms between these two species are probably differences in spawning period combined with well-developed differences in ecological preference. The rôle of sexual selection is not known. The two species will mate in the laboratory.

Summary.—Although some intraspecific crosses in *Rana pipiens* lead to hybrid inviability, every strain of this species tested can be successfully hybridized with *Rana palustris*.

Acknowledgment.—These investigations were aided by a grant from the Penrose Fund of the American Philosophical Society.

[1] Moore, J. A., *Genetics*, **31**, 304–326 (1946).
[2] Moore, J. A., *Jour. Exp. Zoöl.*, **86**, 405–422 (1941).
[3] Moore, J. A., *Genetics*, **27**, 408–416 (1942).
[4] Moore, J. A., *Jour. Heredity*, **34**, 2–7 (1943).
[5] These stages are described in: Pollister, A. W., and Moore, J. A., *Anat. Rec.*, **68**, 489–496 (1937).
[6] Hutchinson, J. B., *Jour. Genetics*, **25**, 281–291 (1932).
[7] Silow, R. A., *Ibid.*, **42**, 259–358 (1941).